Reference: *Biol. Bull.* **188:** 23–31. (February/March, 1995)

Isolation of Biologically Functional RNA During Programmed Death of a Colonial Ascidian

WEN-TEH CHANG[1] AND ROBERT J. LAUZON[1,2,*]

[1]*Department of Microbiology, Immunology and Molecular Genetics, and* [2]*Department of Pediatrics, Albany Medical College, Albany, New York 12208*

Abstract. The blastogenic (asexual) cycle of the colonial ascidian *Botryllus schlosseri* (Tunicata, Ascidiaceae) concludes in a cyclical phase of programmed cell and zooid death called takeover, in which all asexually derived adults die synchronously by apoptosis. The characterization of developmentally regulated genes whose expression patterns are selectively modulated during this process could pave the way to understand how this model organism dies. However, isolation of biologically functional RNA in this and other colonial ascidians with conventional phenol/chloroform-based procedures is hampered by extensive contamination of RNA preparations by pigments. Upon cell lysis, pigments that normally reside within specialized cells in the mantle wall of the adult are released and tightly associate with nucleic acids. Here, we report on the usefulness of a single-step RNA isolation method in which acid guanidinium isothiocyanate is used as an extraction medium, followed by preparative cesium chloride ultracentrifugation. This procedure successfully isolated biologically active, high-purity total RNA (OD_{260}/OD_{280} = 1.9–2.1) from *Botryllus* colonies during takeover, as well as other species of colonial ascidians (*Diplosoma macdonaldii, Botrylloides diegense*) irrespective of pigmentation. Northern blot analysis performed with a ^{32}P-labeled tunicate actin probe detected two polyadenylated transcripts of 1.5 and 1.7 kilobases in length from both growth phase and takeover colonies. Two-dimensional protein gel assays from *in vitro* translated mRNA preparations further revealed that specific transcripts were up-regulated during takeover, while others were repressed or down-regulated. Growth phase and takeover-specific cDNA libraries were constructed from pooled poly(A)$^+$ RNA with a complexity of 1.0×10^7 and 1.2×10^7 recombinants respectively per 100 ng of cDNA before amplification. The procedure described herein renders feasible the cloning of developmentally regulated genes in this organism. In addition, our findings raise the possibility that zooid death in *Botryllus* involves modulated gene expression.

Received 8 July 1994; accepted 22 November 1994.

* Author to whom correspondence should be addressed.

Introduction

Programmed cell death is a fundamental morphogenetic process within developing multicellular animals (Ellis *et al.*, 1991; Schwartz and Osborne, 1993). In adult tissues, cell death also functions as a homeostatic mechanism complementary to mitosis: changes to this balance bring about pathologic abnormalities (Ellis *et al.*, 1991). Recent studies in vertebrates (Owens *et al.*, 1991; Miura *et al.*, 1993; Woronicz *et al.*, 1994; Liu *et al.*, 1994) and invertebrates (Ellis and Horvitz, 1986; Schwartz *et al.*, 1990; White *et al.*, 1994) strongly suggest that cell death is an active process dependent on modulated gene expression. One of the most characteristic forms of cell death is a dynamic morphological process known as apoptosis, characterized by nuclear chromatin condensation and margination, cellular fragmentation into membrane-bounded bodies followed by engulfment and digestion within phagocytic cells (Kerr, 1972).

The colonial ascidian *Botryllus schlosseri* contributes a unique perspective to the study of cell death: adult colonies, derived from a chordate tadpole through palleal budding and which at peak size consist of approximately 1000 asexually derived clones (zooids), undergo weekly phases of regression (Milkman, 1967). Every 5 days at 21°C, the blastogenic (asexual) cycle concludes in a phase of programmed cell and zooid death called takeover, during which all zooids, each containing a functional heart, nervous and digestive systems simultaneously die by an

apoptotic process over a 24-h period and are replaced by a new asexual generation of zooids (Lauzon *et al.*, 1993).

Because the temporal and morphological events of takeover can be predicted in detail in *Botryllus,* we have undertaken a multidisciplinary investigation of the molecular mechanisms underlying programmed death in this model organism. Thus far, molecular studies have been impeded by the lack of appropriate methods for isolation of biologically active mRNA. Unfortunately, *Botryllus* and other colonial ascidians harbor polyphenolic and DOPA-containing pigments that bind tightly with nucleic acids following cellular lysis with detergents and chaotropic agents, thus interfering with the isolation process as well as its subsequent analysis and cloning (Kumar *et al.*, 1988). Moreover, isolation of intact RNA molecules from regressing tissues may also prove to be difficult because, during cell death, a substantial fraction of the RNA pool is rapidly degraded through the enhanced activity of ribonucleases (Cidlowski, 1982; Owens *et al.*, 1991). Therefore, to ensure isolation of biologically active RNA from these organisms, a strategy had to be developed that would eliminate both ribonuclease activity and pigments.

Here, we report on the success of a procedure by which biologically functional RNA suitable for cDNA cloning and other molecular applications can be rapidly isolated from various colonial ascidian species, including *Botryllus.* In addition, we present evidence which indicates that changes in gene expression occur during takeover.

Materials and Methods

Animals

Ascidians (*Botryllus schlosseri, Botrylloides diegense, Diplosoma macdonaldii, Molgula manhattensis*) were collected on glass microscope slides contained within wooden enclosures submerged in the Eel Pond (Woods Hole, MA) and Monterey Bay (CA). They were subsequently maintained in a refrigerated aquarium (150-gallon capacity) containing artificial sea salts, trace and bio-elements (hw-marine mix: Hawaiian Marine Imports Inc., Houston, TX), and were continuously fed with an algal scrubber irradiated for 12 h each day with two 15-watt Aurora 50:50 bulbs (Fritz Pet Products, Dallas, TX). Individual *Botryllus* colonies were developmentally staged with the use of a stereomicroscope (Stemi SV 6, Carl Zeiss, Germany). Following removal of debris and encrusting organisms, all animals were subsequently snap-frozen in liquid nitrogen and stored at −70°C until needed.

RNA extraction

RNA was extracted using a modification of the method by Chirgwin *et al.* (1978). Individual colonies (0.5–1.0 g) were initially ground to a fine powder with liquid nitrogen in a precooled mortar and pestle, and subsequently homogenized in 2.0 ml of a lysis solution containing the following components: 4 *M* guanidium isothiocyanate (Gibco/BRL, Gaithersburg, MD) predissolved in a 0.75 *M* sodium citrate solution (pH = 7.0; 25 m*M* final concentration), 0.2 *M* sodium acetate (pH = 5.0), and 0.1 *M* β-mercaptoethanol. Following transfer of the lysate to a 50-ml polypropylene tube, the DNA was sheared with a 23-gauge needle and syringe, and sodium lauryl sarcosinate (10% stock) was added to a final concentration of 0.5%. The homogenate was incubated on ice for 15 min and centrifuged at 3500 × *g* for 5 min at 4°C. The supernatant was layered on a 1.3-ml cesium chloride (CsCl; Boehringer Mannheim, Indianapolis, IN) solution (5.7 *M* CsCl, 0.5 *M* EDTA, pH = 8.0) in a 13- × 51-mm ultracentrifuge polyallomer tube (Beckman Instruments Inc., Palo Alto, CA), and centrifuged at 40,000 rpm at 20°C for 12 h in an SW50.1 rotor (Beckman Instruments Inc.). Following centrifugation, the RNA pellet was resuspended in diethylpyrocarbonate (DEPC)-treated water (Sigma Chemical Co., St. Louis, MO), precipitated overnight at −20°C in 100% ethanol, and washed in 70% ethanol. The pellet was subsequently dried under vacuum at room temperature, resuspended in DEPC-treated water, and stored at −70°C. For phenol/chloroform-based extractions, the method described by Chomcynski and Sacchi (1987) was used.

Spectrophotometric analysis

An aliquot from each RNA preparation (1-5 μl) was diluted into 250 μl of DEPC-treated water, transferred to a quartz cuvette, and scanned between 240 and 320 nm with a Perkin-Elmer λ-2 microprocessor-controlled spectrophotometer (Perkin-Elmer Inc., Foster City, CA). A GeneQuant spectrophotometer (Pharmacia, Piscataway, NJ) was used to determine total RNA concentrations as outlined in Sambrook *et al.* (1989). During the course of our studies, we observed that OD_{260}/OD_{280} ratios were greatly affected by pH. For instance, DEPC-treated water samples that still contained residual levels of DEPC following autoclaving (pH = 5.0) gave aberrant OD_{260} absorbance readings (between 1.3 and 1.5). Consequently, we routinely autoclaved all our DEPC-treated solutions twice for 30 min each. Poly-A^+ RNA was isolated with the poly-A tract mRNA isolation system from Promega (Promega Corp., Madison, WI), and concentrations were determined with the Dipstick kit by Invitrogen (Invitrogen Corp., San Diego, CA). Both were used according to the manufacturer's specifications.

Northern blot hybridization

Ten micrograms of total RNA was denatured in formaldehyde, size-fractionated in 1% agarose/formaldehyde gels (4 V/cm), and transferred onto nitrocellulose mem-

branes (Schleicher and Schuell, Keene, NH) with 20× SSC. Blots were hybridized overnight with a cytoactin cDNA probe from *Styela clava* (SpCA8; Beach and Jeffery, 1990) under high-stringency conditions (1 *M* NaCl, 10% dextran sulfate, 1% SDS, 100 μg/ml denatured salmon sperm DNA) at 65°C. The SpCA8 probe was labeled by random hexadeoxynucleotide priming to a specific activity of 10^9 cpm/μg DNA (Feinberg and Vogelstein, 1983). Blots were washed three times in 1× SSC (prepared from a 20× SSC stock solution: 3.0 *M* sodium chloride, 0.3 *M* sodium citrate, pH = 7.0) and 0.1% SDS at 65°C 5 min each, followed by two additional washes in 0.1× SSC and 0.1% SDS (65°C) 15 min each, and autoradiographed with Kodak XAR film.

Two-dimensional protein gel electrophoresis

Poly-A^+ RNA (0.2 μg/sample) was translated *in vitro* with a rabbit reticulocyte lysate from Promega using ^{35}S-methionine. Protein products were analyzed by two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975; O'Farrell *et al.*, 1977) on a protein II xi slab cell (Bio-Rad, San Diego, CA). *In vitro*-labeled samples were focused with wide range ampholytes (pH = 3-10; Bio-Lyte 3/10, Bio-Rad) in 4 *M* urea and 10% NP-40, and size-fractionated on 10% sodium-dodecyl-sulfate polyacrylamide gels (SDS-PAGE) along with Brome Mosaic Virus (BMV) molecular weight markers (110, 97, 35 and 20 Kd) provided as a control with *in vitro* translation kits (Promega). Gels were fixed in methanol/acetic acid, enhanced with RESOLUTION (EM Corp, Chestnut Hill, MA), dried under vacuum, and autoradiographed with Kodak XAR film at −70°C. All samples were run at least twice to ensure reproducibility of translational profiles observed by autoradiography.

cDNA library construction

cDNA was synthesized from poly-A^+ RNA of pooled colonies isolated at onset and early stages of takeover (stages D-1 and D-2) or from representative growth stages (A, B-1, B-2 and C-1) with the unizap cDNA synthesis kit from Stratagene (La Jolla, CA) using ^{32}P-dATP. The cDNA products were ligated to *Eco* RI linkers, restricted with *Xho* I, and cloned unidirectionally into lambda zap vector, according to the manufacturer's specifications. Size range of first and second strand cDNA products was determined by alkaline agarose gel electrophoresis by the slide technique. Briefly, 10 ml of 1% molten alkaline agarose (containing 1 ml of 10× alkaline agarose buffer: 3 ml of 5 N NaOH, 2 ml of 0.5 M EDTA and 45 ml of sterile milli-Q water) was added near the upper center of a 5- × 7.5-cm glass slide, to which a mini-gel comb had been attached over it with high-tension clips. The gel was run in 1× alkaline buffer at 75 V for 2 h at room temperature. Following electrophoresis, the gel was blotted dry with several changes of Kimwipes EX-L (Kimberly-Clark, Roswell, GA), sealed in an air-tight hybridization bag, and autoradiographed with Kodak XAR film at room temperature. The library was packaged and titered according to Stratagene's specifications. The level of non-recombinants was determined by plating various phage dilutions with XL1-Blue MRF' cells along with IPTG (200 mg/ml in water) and X-gal (20 mg/ml in dimethylformamide). For either takeover or growth phase cDNA library, blue background plaques were not observed on plates containing up to 10^3 PFUs (plaque forming units), indicating that the percentage of non-recombinants was very low (less than 1×10^5 PFUs/μg of phage arms). Lastly, the primary library was amplified in XL1-Blue, phage suspensions were stored at −70°C, and an aliquot was prepared to assess the quality of the cDNA library. The quality of each cDNA library was assayed by probing nitrocellulose plaque lifts for representation of actin-complementary sequences using the SpCA8 cytoactin cDNA clone. Phage transfer was performed for 1 min at room temperature, and filters were sequentially placed for 3 min each onto sheets of 3MM paper saturated with the following solutions: (1) 0.5 *N* sodium hydroxide and 1.5 *M* sodium chloride, (2) 10% SDS, (3) 0.5 *M* Tris-HCl pH = 8.0 and 1.5 *M* NaCl, and (4) 2× SSC. Membranes were subsequently baked at 80°C under vacuum for 30 min, hybridized with the ^{32}P-labeled SpCA8 cDNA clone, and autoradiographed with XAR film at −70°C. Hybridization conditions and post-hybridization washes were identical to those used in the northern blot analysis.

Results

The blastogenic cycle of B. schlosseri

Developmental staging of *B. schlosseri* colonies was adapted from the nomenclature used by Mukai and Watanabe (1976), as well as Izzard (1973), and is described in Table I and depicted in Figure 1. Following metamorphosis of the free-swimming tadpole, a colony arises by weekly cycles of palleal budding, in which the bud evaginates from the wall of its parent zooid. Under optimal growth conditions, two to three primary buds are produced per zooid and can be easily observed dorsally by stage B-2 (Fig. 1, panel B). By stage C-1, organogenesis begins in the secondary bud with the formation of primary atrial folds, and at this time it exhibits an elongated appearance as primary organs (gut rudiment) begin to form (not shown). At 21°C, the cycle concludes on the fifth day with the synchronous death of all parent zooids, a process called takeover (Lauzon *et al.*, 1992). The onset of takeover is characterized by the shutdown of both oral and excurrent siphons (Fig. 1, panel C). At this stage, while buds begin to move dorsally, zooids are still re-

Table I

Developmental stages of the blastogenic cycle

Stage	Characteristic
A	Onset of new cycle; opening of oral and excurrent siphons.
B-1	Secondary bud skewing to parent zooid's anterior hemisphere. Heartbeat begins in primary bud.
B-2	Secondary bud is a closed double-layered vesicle.
C-1	Organogenesis (atrial folds) begins in secondary bud. Secondary bud elongates along its anteroposterior axis.
C-2	Primary subdivisions completed in secondary bud.
D-1	Onset of takeover: shutdown of zooid's oral and excurrent siphons. Primary buds move dorsally.
D-2	Early takeover; contraction of zooid along its anteroposterior axis.
D-3	Mid-takeover (zooid involution): visceral organs are being resorbed. Apoptotic cell death and macrophage phagocytosis are prevalent.
D-4	End of takeover: cessation of heartbeat in zooid. Siphons of new asexual generation not yet open.

sponsive to mechanical stimulus. In the early stages of takeover (3–5 h post-onset), the zooids contract along their anteroposterior axis and begin to shrink in size (Fig. 1, panel D). Pigment cells, which normally reside in the zooid's mantle wall, begin to accumulate in the vascular ampullae. In the middle stages of takeover (12–15 h post-onset), visceral organs die principally through an apoptotic process (Lauzon *et al.*, 1993), although necrotic changes can also be observed alone or in combination with an apoptotic morphology. Takeover concludes with the cessation of heartbeat in zooids, and a new cycle begins with the opening of siphons in the next asexual generation of zooids (panel A).

RNA isolated by preparative ultracentrifugation is biologically functional

As shown in Figure 2A, when RNA was extracted with the guanidine isothiocyanate/phenol/chloroform procedure (Chomcynski and Sacchi, 1987), the spectrophotometric absorbance pattern was severely disrupted, exhibiting a peak absorbance at 268 nm instead of 260 nm. All preparations extracted in this manner were significantly contaminated with blue and red pigments that could not be removed upon further phenol/chloroform extraction. In addition, the yields from these preparations were very poor (10–20 μg of total RNA/g tissue), and often displayed OD_{260}/OD_{280} ratios greater than 2.5, suggesting that pigments were contributing to the altered ratios. Furthermore, when the preparations were size-fractionated on formaldehyde/agarose gels, most of the sample remained in the loading well. We surmise that since many pigments have been reported to be polyphenolic in nature (Kumar *et al.*, 1988), RNA was most likely sequestered to the organic phase along with them. In contrast, RNA isolated by means of cesium chloride ultracentrifugation was spectrophotometrically pure (Fig. 2B), exhibited optimal OD_{260}/OD_{280} ratios between 1.9 and 2.1, and consistently produced yields ranging between 0.5 and 0.8 μg/mg of colony. When cesium-chloride-purified samples were electrophoresed, prominent 28S and 18S ribosomal RNA bands were visualized irrespective of the developmental stage of the colony (Fig. 3, lanes 2, 3, 4, 7, 8) or species (Fig. 3, lanes 5, 6). To test the integrity of the RNA, samples from various ascidians were size-fractionated by agarose/formaldehyde gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized to a ^{32}P-labeled cytoactin cDNA probe from *Styela clava*. The results, which are shown in Figure 4, indicate that all colonial ascidians (*Botryllus*, *Botrylloides*, and *Diplosoma*) expressed two polyadenylated transcripts of 1.5 and 1.7 kb in length (panel A, lanes 1–4; panel B, lane 5). In contrast, the solitary ascidian *Molgula manhattensis* expressed only a single 1.5-kb transcript. Furthermore, both transcripts were expressed during all stages of the blastogenic cycle in *Botryllus* (Fig. 4, panel B).

We next sought to determine whether *Botryllus* RNA isolated by this method could also be *in vitro*-translated into protein. Samples (0.2 μg) of poly-A$^+$ RNA from various developmental stages were translated with a rabbit reticulocyte lysate with ^{35}S-methionine and analyzed by two-dimensional polyacrylamide gel electrophoresis to examine patterns of gene expression between different stages of the blastogenic cycle. The results (Fig. 5) demonstrate that RNA preparations could be successfully translated and focused into a wide spectrum of acidic- and basic-range polypeptides during both the growth phase of the cycle and takeover. Furthermore, at the onset of a new blastogenic cycle, several different spots were identified from the acidic and basic range that were absent in the early stages of takeover (panel A). Additional transcripts (for instance, arrow in panels A and B, Fig. 5) appeared to be significantly down-regulated during takeover. Conversely, other transcripts were expressed in the early stages of takeover, but absent at the beginning of a new asexual cycle (panel B) or other stages (not shown).

Lastly, in order to determine whether mRNA from takeover colonies was suitable for cDNA synthesis, poly-A$^+$ RNA was reverse-transcribed, and cDNA products were subsequently size-fractionated by alkaline gel electrophoresis. The results (Fig. 6) indicate that both first- and second-strand cDNA products exhibited an appropriate size range, with the bulk distributed between 300 bases and 2.5 kb. Pooled cDNA products were then used to construct a unidirectional library into lambda zap. The size of the primary nonamplified library was 1.2×10^7 PFUs/100 ng of cDNA, as determined from

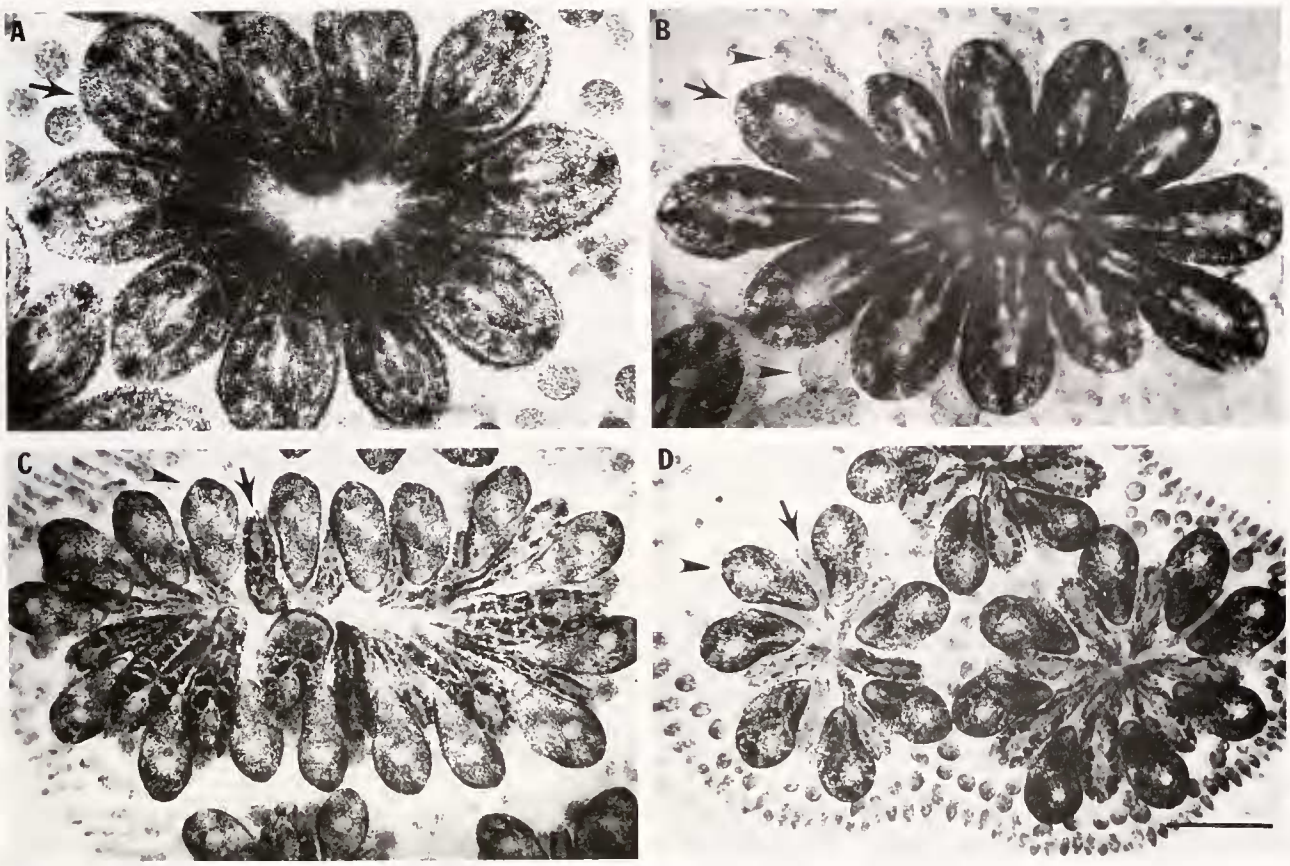

Figure 1. The blastogenic cycle of *Botryllus schlosseri*. Individual colonies were developmentally staged by stereomicroscopy and are depicted dorsally in panels A through D. Panel A shows a colony at the onset of a new cycle. Note that the primary buds are not visible from the dorsal plane. Panel B shows a colony during stage B-2 (see Table 1 for specific details of individual stages), in which primary buds are now visible. The onset of takeover (panel C) is characterized by the shutdown of oral and excurrent siphons in all zooids (arrow) and star-shaped systems. Buds (arrowhead) have begun their dorsal migration. In the early stages of takeover (panel D), each zooid undergoes a synchronous polarized contraction along its anteroposterior axis. Zooid regression is completed in approximately 24 h at 21°C, and a new cycle begins with the opening of siphons from the new asexual generation of zooids. Bar represents 1 mm.

plaque counts using serial dilutions of phage suspensions. A cDNA probe encoding cytoplasmic actin from *Styela clava* (as a prototype abundant sequence) was then used to screen nitrocellulose plaque lifts to ensure adequate representation of this sequence. A comparable screen was performed with a growth phase (pooled stages A, B-1 and C-1) cDNA library (1.0×10^7 PFUs nonamplified). The percentages of actin-positive clones (Fig. 7) were found to be comparable in both libraries, namely 3.0% (150 positive/5×10^3 plaques) in the takeover and 3.2% in the growth phase cDNA library (58 positive/1.8×10^3 plaques).

Discussion

The findings presented in this paper indicate that RNA isolated by cesium chloride ultracentrifugation is optimally suited for a wide range of molecular applications, including northern blot analysis, *in vitro* translation, and cDNA synthesis from dying tissues of *Botryllus schlosseri* and other colonial ascidians. Because colonial ascidians (Kumar *et al.*, 1988) and other marine invertebrates (Groppe and Morse, 1993) exhibit a spectacular range of pigmentation patterns, they have been reported to pose a distinct problem in the isolation of nucleic acids. Polyphenolic compounds and DOPA-containing proteins, which interfere with nucleic acid isolation, have been found in the adult tunic and mantle wall of both solitary and colonial ascidians (Kumar *et al.*, 1988). In *Botryllus* colonies found on the eastern coast of the United States, the problem is intensified because most colonies contain an alcohol-insoluble red pigment that cannot be removed from nucleic acids with conventional lysis buffers followed by phenol/chloroform-based extractions. The addition of

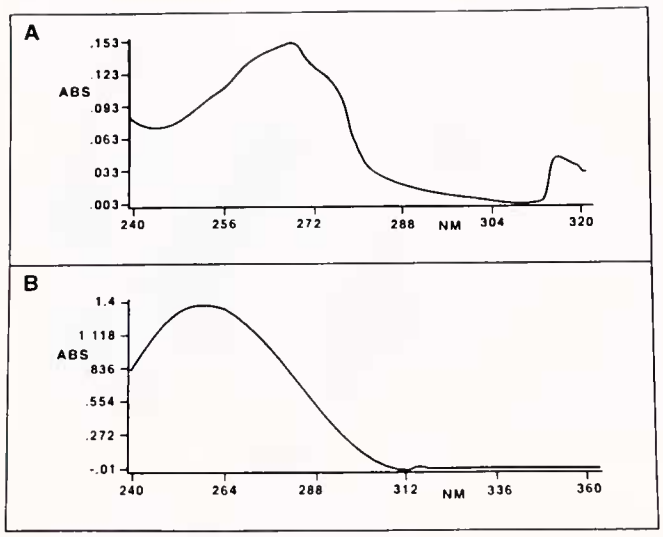

Figure 2. Spectrophotometric scanning analysis of isolated total RNA from *Botryllus schlosseri*. (A) RNA isolated from *B. schlosseri* with a conventional extraction method that utilizes phenol/chloroform/guanidine isothiocyanate (Chomcynski and Sacchi, 1987) demonstrates an altered ultraviolet absorption spectrum. In contrast (B), RNA isolated with the single-step cesium-chloride method is free of contaminants and exhibits an optimal OD_{260}/OD_{280} ratio (1.9–2.1).

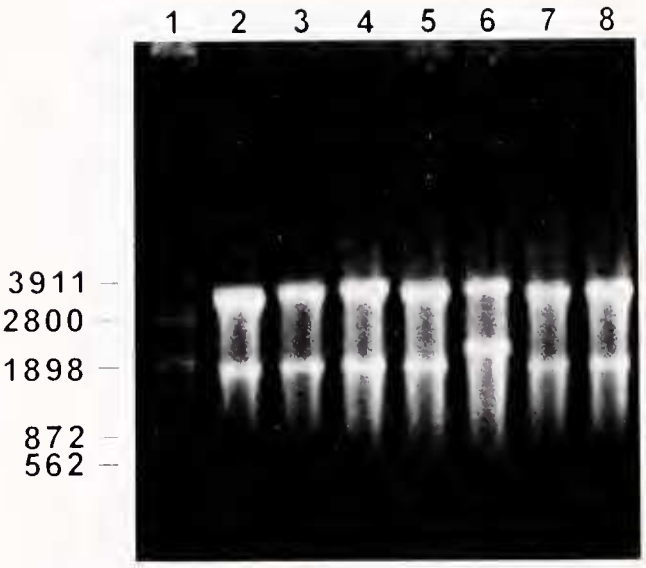

Figure 3. Ethidium bromide staining of total RNA isolated by preparative ultracentrifugation. Lane 1, RNA markers. Lanes 2, 3, 7, and 8, *Botryllus schlosseri* from Eel Pond (Woods Hole, MA): lane 2 (stage A), lane 3 (stage B-2), lane 7 (early takeover; 3 h post-onset), and lane 8 (mid-takeover, 12 h post-onset). Lane 4, *B. schlosseri* from Monterey Bay, CA (stage C-2). Lane 5, *Botryllodes diegense* (growth phase). Lane 6, *Diplosoma macdonaldii*.

a cesium chloride ultracentrifugation step permitted the recovery of high yields of spectrophotometrically and electrophoretically pure RNA preparations, irrespective of pigmentation or species. Groppe and Morse (1993) recently described a two-step cold method of isolating RNA from *Haliotis rufescens* (red abalone); the method provided high yields of pigment-free, undegraded material suitable for cDNA cloning. The first step, a phenol/chloroform extraction performed at 0°C, was crucial for the removal of ribonuclease activity, and the second step, employing ultracentrifugation through a cesium chloride gradient, removed an inhibitor of reverse transcriptase. The observations reported herein indicate that in *Botryllus* and other colonial ascidians, only a single preparative ultracentrifugation step through cesium chloride is required for isolation of biologically functional RNA.

However, our findings are in contrast with those of Kumar *et al.* (1988), who reported that they successfully isolated RNA from various ascidians by using only a phenol/chloroform-based procedure. In our hands, all preparations isolated using phenol/chloroform were significantly contaminated with pigments and gave very poor yields. Furthermore, much of the original sample was left in the loading well during formaldhyde/agarose gel electrophoresis, and the efficiencies for *in vitro* translation reactions and reverse-transcription for cDNA library construction were significantly impaired (W-T.C and R.J.L., unpub. obs.). At present, we have no explanation for the discrepancy between our results and those of Kumar *et al.* (1988). One possibility is that the composition of pigments found in *Botryllus* and other botryllid ascidians may differ from those found in other solitary or colonial species reported

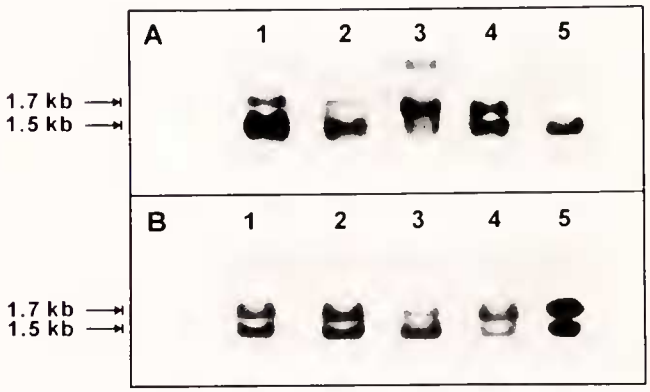

Figure 4. Northern blot analysis of RNA isolated from various species of colonial and solitary ascidians. Samples were electrophoresed on a 1% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled cytoactin probe SpCA8 from *Styela clava* (Beach and Jeffery, 1990). (A) Lane 1, *Botryllus schlosseri* (stage A) from Eel Pond (Woods Hole, MA); lane 2, *B. schlosseri* (stage C-2) from Monterey Bay (CA); lane 3, *Botrylloides diegense* (growth phase); lane 4, *Diplosoma macdonaldii*; lane 5, *Molgula manhattensis*. (B) Total RNA samples of *B. schlosseri* from Eel Pond isolated at various stages of the blastogenic cycle (lanes 1–4), and pooled poly-A$^+$ RNA from stages A–C colonies (lane 5). Lane 1, early takeover; lane 2, mid-takeover; lane 3, stage A; lane 4, stage B-1.

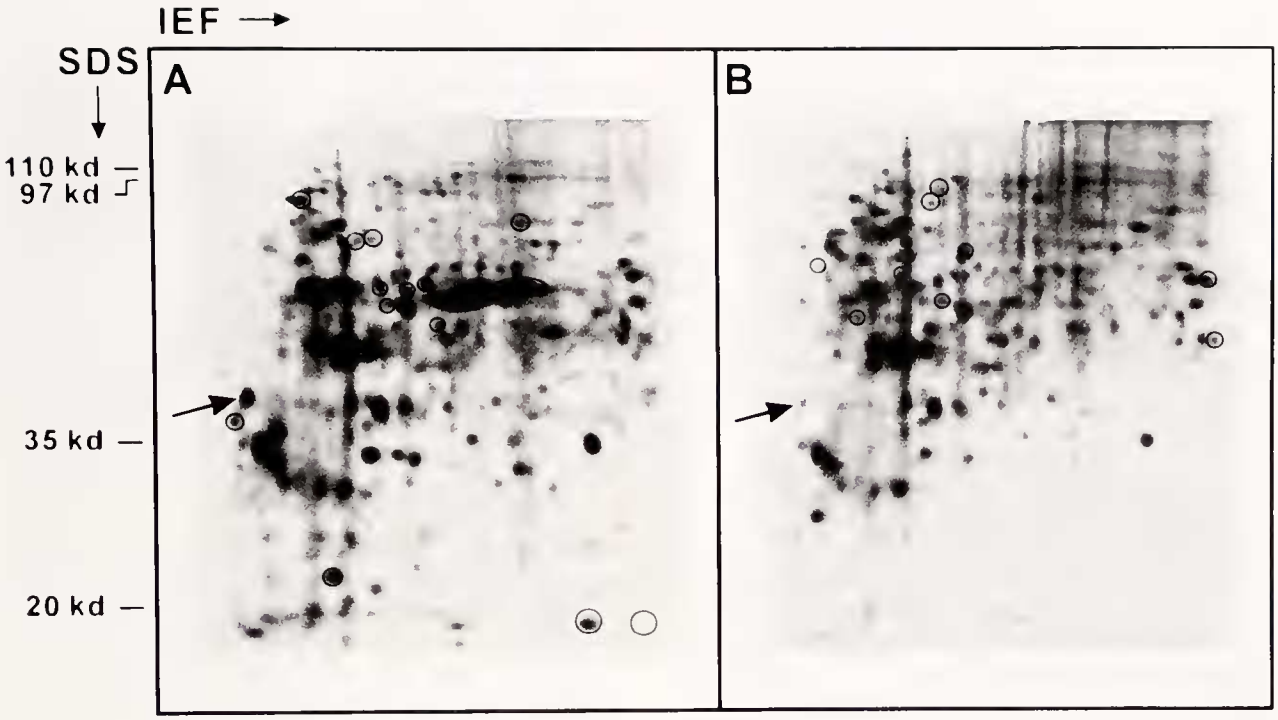

Figure 5. Two-dimensional protein gel analysis of *in vitro*-translated RNA with ^{35}S-methionine reveal changes in gene expression during takeover. Panel A depicts a colony at the onset of a new blastogenic cycle (stage A), whereas panel B is from a colony in early takeover (3 h post-onset). The circled spots in panel A represent transcripts that are repressed in the early stages of takeover. The arrows in panels A and B depict a representative polypeptide whose mRNA is down-regulated during takeover. Conversely, the circled spots in panel B are transcripts that appear to be induced *de novo* during takeover. Abbreviations: SDS, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) dimension; IEF, isoelectric focusing dimension.

by Kumar *et al.* (1988). The presence of contaminating pigments markedly altered $OD_{260/280}$ ratios, presumably by absorbing in the range that is optimal for nucleic acids (*i.e.*, 260 nm). In support of this hypothesis, we have recently observed that pigment cells from live colonies are fluorescent under ultraviolet light (R.J.L., unpub. obs.).

Northern blot analysis with cesium-chloride-purified material further revealed that the RNA was not degraded by ribonuclease activity present during zooid regression. Previous studies have cautioned that isolation of intact RNA molecules from dying tissues can be significantly impeded by ribonucleases (Cidlowski, 1982; Owens *et al.*, 1991). In addition, all colonial ascidian species reported in this paper (*Botryllus schlosseri* from the East and West coasts, *Botryloides diegense*, and *Diplosoma macdonaldii*) expressed two poly-A^+ transcripts of 1.5 and 1.7 kb in length that hybridized to a cytoplasmic cDNA clone from *Styela clava*. This was in contrast to the single 1.5 kb-message found in the solitary ascidian *Molgula manhattensis*. The significance of two mRNAs in colonial ascidians is unclear, although another solitary tunicate, *Styela clava*, was previously reported to express a single 1.8-kb message during both embryonic and post-metamorphic development (Beach and Jeffery, 1990). The additional 1.7-kb band in colonial species may represent a cross-hybridizing muscle actin transcript. Tomlinson *et al.* (1987) showed that a probe made exclusively from the 3′ untranslated region of a *Styela* muscle actin clone detected transcripts exclusively in muscle cells, whereas one made from the coding region, such as the cytoplasmic cDNA clone used in this study (*e.g.*, SpCA8), detected both muscle and nonmuscle transcripts. However, several lines of evidence argue against this scenario. First, although both transcripts were expressed at all phases of the blastogenic cycle in *Botryllus* including takeover, the relative intensity of the bands varied at different stages of the cycle. Second, if the 1.7-kb transcript represented a cross-reactive muscle mRNA, one would expect the intensity of the hybridizing band to be less than the 1.5-kb transcript at any given time under high-stringency conditions. This condition was clearly not observed. Alternatively, both transcripts could result from alternative splicing. The expression of an additional 1.7-kb transcript could be functionally related to the colonial life style, but seems unlikely to be associated

with zooid death since *Diplosoma* species do not undergo takeover. An intriguing possibility is that it may be expressed during bud development. Therefore, determination of the complete nucleotide sequences of both cDNA clones followed by *in situ* hybridization with non-cross-hybridizing probes will be required to resolve this issue.

Studies with invertebrate (Wadewitz and Lockshin, 1988) and vertebrate (Wang and Brown, 1991) developmental systems indicate that individual death programs may involve fewer than 40 up-regulated genes. For instance, thyroid-hormone-mediated changes leading to tail resorption in *Xenopus laevis* involve two periods of gene expression during which all genes belonging to a specific group are induced with identical kinetics. Conversely, about 10 additional genes are down-regulated with identical decay kinetics (Wang and Brown, 1993). These observations indicate that in amphibians the death program reflects a relatively simple pattern of gene expression. The initial findings reported here with two-dimensional protein gels from *Botryllus* suggest that modulated gene expression occurs during the takeover phase of blastogenesis. We have previously demonstrated that takeover involves the polarized breakdown of the perivisceral extracellular matrix along the zooid's anteroposterior axis, followed by apoptotic and necrotic morphological changes within dying visceral tissues (Lauzon *et al.*, 1992, 1993). Changes in gene expression may thus be associated with these morphological events. Unfortunately, the shutdown of oral siphons during takeover precluded us from analyzing ^{35}S-methionine incorporation patterns *in vivo*. Therefore, the possibility cannot be ruled out that differences in 2-D protein profiles between the onset of blastogenesis and takeover are due to inherent limitations of the *in vitro* translation kits. In addition, since clonal replicates were not used in any of these studies, the differences observed may represent intra-species polymorphisms. Lastly, since takeover involves the simultaneous regression of adult zooids along with asexual growth of the future parental generation, the possibility cannot be excluded that transcriptional changes also occur in buds or in the colonial vasculature. Therefore, assessing the specificity of transcriptional changes will require isolation of takeover-specific mRNAs and analysis of their spatial distribution pattern by *in situ* hybridization. We are currently using differential mRNA display (Liang and Pardee, 1992) as a means for ultimately characterizing full-length transcripts from the cDNA libraries. Interestingly, the percentages of actin-positive PFUs were similar in the growth phase and takeover libraries (3.2 versus 3.0). Libraries with reported actin cDNA-positive frequencies above 0.1% have yielded clones of interest for sequences of moderate to low abundance, whereas percentages below 0.05% have not (Hagen *et al.*, 1988). Collectively, our findings strongly suggest that both libraries are likely to contain cDNAs corresponding to single-copy gene transcripts. The characterization of genes involved in zooid regression could provide a fundamental understanding of molecular mechanisms of programmed cell death in *Botryllus* and other metazoans.

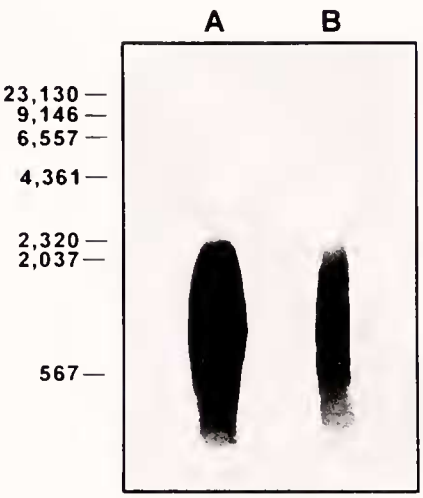

Figure 6. Alkaline agarose gel electrophoresis assay of first- and second-strand cDNA synthesis in *Botryllus schlosseri*. First- (lane A) and second-strand (lane B) cDNA products were converted using pooled poly-A$^+$ RNA isolated from colonies during takeover (onset and early takeover). Note that both lanes exhibit a broad size distribution of cDNA products, with the majority of material ranging between 1 and 2 Kb.

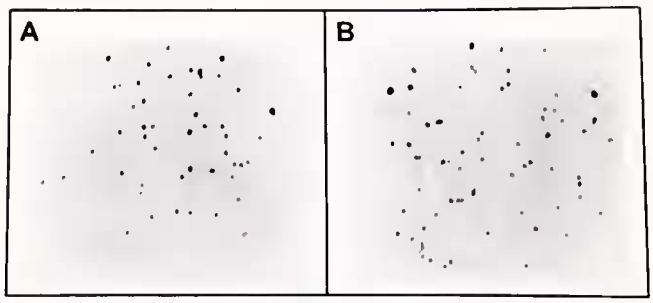

Figure 7. Nitrocellulose plaque lifts from growth stages (panel A) and takeover (panel B) cDNA libraries hybridized with an ascidian ^{32}P-labeled cytoactin probe from *Styela clava* (SpCA8). Percent positive plaque forming units in (B) was 3.0% (150 positive out of 5×10^3 PFUs) compared to 3.2% for the growth phase cDNA library (58 positive out of 1.8×10^3 PFUs), indicating that actin was adequately represented.

Acknowledgments

The authors gratefully acknowledge Dr. Craig Tomlinson for his generous gift of the SpCA8 cytoactin cDNA clone, and two anonymous reviewers for their efforts in improving the focus of this manuscript. This work was supported by a Basil O'Connor Starter Scholar Award from the March of Dimes Birth Defects Foundation #5-

FY94-0813, and from a Frederick Bang Fellowship at the Marine Biological Laboratory, Woods Hole, Massachusetts.

Literature Cited

Beach, R. L., and W. R. Jeffery. 1990. Temporal and spatial expression of a cytoskeletal actin gene in the ascidian *Styela clava*. *Devel. Genet.* **11:** 2–14.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1978. Isolation of biologically active ribonucleic acid from sources enriched by ribonuclease. *Biochemistry* **18:** 5294–5299.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem* **162:** 156–159.

Cidlowski, J. A. 1982. Glucocorticoids stimulate ribonucleic acid degradation in isolated rat thymic lymphocytes *in vitro*. *Endocrinology* **111:** 184–190.

Ellis, H. M., and H. R. Horvitz. 1986. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44:** 817–829.

Ellis, R. E., J. Yuan, and H. R. Horvitz. 1991. Mechanisms and functions of cell death. *Ann. Rev. Cell Biol* **7:** 663–698.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6–13.

Groppe, J. C., and D. E. Morse. 1993. Isolation of full-length RNA templates for reverse transcriptase from tissues rich in RNase and proteoglycans. *Anal. Biochem* **210:** 337–343.

Hagen, F. S., C. L. Gray, and J. L. Kuijper. 1988. Assaying the quality of cDNA libraries. *Biotechniques* **6:** 340–345.

Izzard, C. S. 1973. Development of polarity and bilateral asymmetry in the palleal bud of *Botryllus schlosseri* (Pallas). *J. Morph.* **139:** 1–26.

Kerr, J. F. R., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J Cancer* **26:** 239–257.

Kumar S., B. M. Degnan, I. L. Ross, C. J. Hawkins, and M. F. Lavin. 1988. Isolation of DNA and RNA from ascidians. *Mar. Biol.* **98:** 95–100.

Lauzon, R. J., K. J. Ishizuka, and I. L. Weissman. 1992. A cyclical, developmentally-regulated death phenomenon in a colonial urochordate. *Dev. Dyn.* **194:** 71–83.

Lauzon, R. J., C. W. Patton, and I. L. Weissman. 1993. A morphological and immunohistochemical study of programmed cell death in *Botryllus schlosseri*. *Cell Tis. Res.* **272:** 115–127.

Liang, P., and A. B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257:** 967–970.

Liu, Z-G., S. W. Smith, K. A. McLaughlin, L. M. Schwartz, and B. A. Osborne. 1994. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* **367:** 281–284.

Milkman, R. 1967. Genetic and developmental studies on *Botryllus schlosseri*. *Biol. Bull.* **132:** 229–243.

Miura, M., H. Zhu, R. Rotello, E. A. Hartwieg, and J. Yuan. 1993. Induction of apoptosis in fibroblasts by IL-1-beta-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene ced-3. *Cell* **75:** 653–660.

Mukai, H., and H. Watanabe. 1976. Studies on the formation of germ cells in a compound ascidian *Botryllus primigenus* Oka. *J. Morphol.* **148:** 337–362.

O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250:** 4007–4021.

O'Farrell, P. Z., H. M. Goddman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12:** 1133–1142.

Owens, G. P., W. E. Hahn, and J. J. Cohen. 1991. Identification of mRNAs associated with programmed cell death in immature thymocytes. *Molec. Cell. Biol.* **11:** 4177–4188.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning—A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory. Cold Spring Harbor Lab Press, Cold Spring Harbor, NY.

Schwartz, L. M., L. Kosz, and B. K. Kay. 1990. Gene activation is required for developmentally programmed cell death. *Proc. Natl. Acad. Sci. USA* **87:** 6594–6598.

Schwartz, L. M., and B. A. Osborne. 1993. Programmed cell death, apoptosis and killer genes. *Immunol. Today* **14:** 582–590.

Tomlinson, C. R., R. L. Beach, and W. R. Jeffery. 1987. Differential expression of a muscle actin gene in muscle cell lineages of ascidian embryos. *Development* **101:** 751–765.

Wadewitz, A. G., and R. A. Lockshin. 1988. Programmed cell death: dying cells synthesize a co-ordinated, unique set of proteins in two different episodes of cell death. *FEBS Letters* **241:** 19–23.

Wang, Z., and D. D. Brown. 1991. A gene expression screen. *Proc. Natl Acad Sci. USA* **88:** 11505–11509.

Wang, Z., and D. D. Brown. 1993. Thyroid hormone-induced gene expression program for amphibian tail resorption. *J Biol. Chem.* **268:** 16270–16278.

White, K., M. E. Grether, J. M. Abrams, L. Young, K. Farrell, and H. Steller. 1994. Genetic control of programmed cell death in *Drosophila*. *Science* **264:** 677–683.

Woronicz, J. D., B. Cain, V. Ngo, and A. Winoto. 1994. Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* **367:** 277–281.